



06-09-03

GP: 1652  
#7  
Dmt  
6-3-03

Express Mail No. EL 451 600 398 US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Markau *et al.*

Confirmation No.: 3877

Serial No.: 10/072,436

Group Art Unit: 1652

Filing Date: February 5, 2002

Examiner: Richard G. Hutson

For: *MODIFIED DNA-POLYMERASE FROM  
CARBOXYDOTHERMUS HYDROGENOFORMANS  
AND ITS USE FOR COUPLED REVERSE  
TRANSCRIPTION AND POLYMERASE CHAIN  
REACTION*

Attorney Docket No.: 1803-332-999

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Respectfully submitted,

Dated: June 5, 2003

  
Rahul Pathak 42,983  
(Reg. No. )  
For: Nikolaos C. George (Reg. No. 39,201)  
**Pennie & Edmonds LLP**  
1155 Avenue of the Americas  
New York, New York 10036  
(212) 790-9090

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**Patentanmeldung Nr. Patent application No. Demande de brevet n°**

**97121151.1**



Der Präsident des Europäischen Patentamts;  
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**Blatt 2 der Bescheinigung**  
**Sheet 2 of the certificate**  
**Page 2 de l'attestation**

Anmeldung Nr.:  
Application no.: 97121151.1  
Demande n°:

Anmeldetag:  
Date of filing: 02/12/97  
Date de dépôt:

Anmelder:  
Applicant(s):  
Demandeur(s):  
Roche Diagnostics GmbH  
68298 Mannheim  
GERMANY

Bezeichnung der Erfindung:  
Title of the invention:  
Titre de l'invention:

Modified DNA-polymerase from carboxydotherrnus hydrogenofomans and its use for coupled reverse transcription and polymerase chain reaction

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat:  
State:  
Pays:

Tag:  
Date:  
Date:

Aktenzeichen:  
File no.  
Numéro de dépôt:

Internationale Patentklassifikation:  
International Patent classification:  
Classification internationale des brevets:  
C12N15/31, C12N9/12, C12Q1/68

Am Anmeldetag benannte Vertragstaaten:  
Contracting states designated at date of filing:  
Etats contractants désignés lors du dépôt:

~~AT/BE/CH/DE/ES/FR/GB/GR/IE/IT/LI/LU/MC/NL/PT/SE~~

Bemerkungen:  
Remarks:  
Remarques:

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11.02.97

Boehringer Mannheim GmbH

4765/00/EP

## Modified DNA-Polymerase from *Carboxydotherrnus hydrogenoformans* and its use for Coupled Reverse Transcription and Polymerase Chain Reaction

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The present invention relates to a modified DNA-polymerase having reverse transcriptase activity and reduced 5'-3' exonuclease activity derived from a native polymerase which is obtainable from *Carboxydotherrnus hydrogenoformans*.

Furthermore the invention relates to the field of molecular biology and provides methods for amplifying a DNA segment from an RNA template using an enzyme with reverse transcriptase activity (RT-PCR). In another aspect, the invention provides a kit for Coupled High Temperature Reverse Transcription and Polymerase Chain Reaction.

Heat stable DNA polymerases (EC 2.7.7.7. DNA nucleotidyltransferase, DNA-directed) have been isolated from numerous thermophilic organisms (for example: Kaledin et al. (1980), *Biokhimiya* **45**, 644-651; Kaledin et al. (1981) *Biokhimiya* **46**, 1576-1584; Kaledin et al. (1982) *Biokhimiya* **47**, 1785-1791; Ruttimann et al. (1985) *Eur. J. Biochem.* **149**, 41-46; Neuner et al. (1990) *Arch. Microbiol.* **153**, 205-207).

For some organisms, the polymerase gene has been cloned and expressed (Lawyer et al. (1989) *J. Biol. Chem.* **264**, 6427-6437; Engelke et al. (1990) *Anal. Biochem.* **191**, 396-400; Lundberg et al. (1991) *Gene* **108**, 1-6; Perler et al. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 5577-5581).

Thermophilic DNA polymerases are increasingly becoming important tools for use in molecular biology and there is growing interest in finding new polymerases which have more suitable properties and activities for use in diagnostic detection of RNA and DNA, gene cloning and DNA sequencing. At present, the thermophilic DNA polymerases mostly used for these purposes are from *Thermus* species like Taq polymerase from *T. aquaticus* (Brock et al. (1969) *J. Bacteriol.* **98**, 289-297)

The term "reverse transcriptase" describes a class of polymerases characterized as RNA-dependent DNA-polymerases. All known reverse transcriptases require a primer to synthesize a DNA-transcript from an RNA template. Historically, reverse transcriptase has been used primarily to transcribe mRNA into cDNA which can then be cloned into a vector for further manipulation.

Reverse transcription is commonly performed with viral reverse transcriptases like the enzymes isolated from *Avian myeloblastosis virus* or *Moloney murine leukemia virus*. Both enzymes mentioned are active in the presence of magnesium ions but have the disadvantages to possess RNase H-activity, which destroys the template RNA during the reverse transcription reaction and have a temperature optimum at 42°C or 37°C, respectively. Avian myeloblastosis virus (AMV) reverse transcriptase was the first widely used RNA-dependent DNA-polymerase (Verma (1977) *Biochem. Biophys. Acta* 473, 1). The enzyme has 5'-3' RNA-directed DNA polymerase activity, 5'-3' DNA directed DNA polymerase activity, and RNaseH activity. RNaseH is a processive 5'-3' ribonuclease specific for the RNA strand of RNA-DNA hybrids (Perbal (1984), *A Practical Guide to Molecular Cloning*, Wiley & Sons New York). Errors in transcription cannot be corrected because known viral reverse transcriptases lack the 3'-5' exonuclease activity necessary for proofreading (Saunders and Saunders (1987) *Microbial Genetics Applied to Biotechnology*, Croom Helm, London). A detailed study of the activity of AMV reverse transcriptase and its associated RNaseH activity has been presented by Berger et al., (1983) *Biochemistry* 22, 2365-2372.

DNA polymerases isolated from mesophilic microorganisms such as *E. coli* have been extensively characterized (see, for example, Bessmann et al. (1957) *J. Biol. Chem.* 233, 171-177 and Buttin and Kornberg (1966) *J. Biol. Chem.* 241, 5419-5427). *E. coli* DNA polymerase I (Pol I) is useful for a number of applications including: nick-translation reactions, DNA sequencing, in vitro mutagenesis, second strand cDNA synthesis, polymerase chain reactions (PCR), and blunt end formation for linker ligation (Maniatis et al., (1982) *Molecular Cloning: A Laboratory Manual* Cold Spring Harbor, New York).



Several laboratories have shown that some polymerases are capable of *in vitro* reverse transcription of RNA (Karkas (1973) *Proc. Nat. Acad. Sci. USA* **70**, 3834-3838; Gulati et al. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 1035-1039; and Wittig and Wittig, (1978) *Nuc. Acids Res.* **5**, 1165-1178). Gulati et al. found that *E. coli* Pol I could be used to transcribe Q $\beta$  viral RNA using oligo(dT)<sub>10</sub> as a primer. Wittig and Wittig have shown that *E. coli* Pol I can be used to reverse transcribe tRNA that has been enzymatically elongated with oligo(dA). However, as Gulati et al. demonstrated, the amount of enzyme required and the small size of cDNA product suggest that the reverse transcriptase activity of *E. coli* Pol I has little practical value.

Alternative methods are described using the reverse transcriptase activity of DNA polymerases of thermophilic organisms which are active at higher temperatures. Reverse transcription at higher temperatures is of advantage to overcome secondary structures of the RNA template which could result in premature termination of products. Thermostable DNA polymerases with reverse transcriptase activities are commonly isolated from *Thermus* species. These DNA polymerases however, show reverse transcriptase activity only in the presence of manganese ions. These reaction conditions are suboptimal, because in the presence of manganese ions the polymerase copies the template RNA with low fidelity.

Another feature of the commonly used reverse transcriptases is that they do not contain 3'-5' exonuclease activity. Therefore, misincorporated nucleotides cannot be removed and thus the cDNA copies from the template RNA may contain a significant degree of mutations.

One of the known DNA polymerases having high reverse transcriptase activity is obtainable from *Thermus thermophilus* (Tth polymerase) (WO 91/ 0994). Tth polymerase, as well as Taq polymerase, lacks 3' to 5' exonucleolytic proofreading activity. This 3' to 5' exonuclease activity is generally considered to be desirable because it allows removal of misincorporated or unmatched bases in the newly synthesized nucleic acid sequences. Another thermophilic pol I-type DNA polymerase isolated from *Thermotoga maritima* (Tma pol) has 3' to 5' exonuclease activity. U.S. patent 5,624,833 provides means for isolating and producing Tma polymerase.

However, both DNA polymerases, Tth as well as Tma polymerase, show reverse transcriptase activity only in the presence of manganese ions.

The DNA polymerase of *Carboxydotherrnus hydrogenoformans* shows reverse transcription activity in the presence of magnesium ions and in the substantial absence of manganese ions and can be used to reverse transcribe RNA, to detect and amplify (in combination with a thermostable DNA polymerase like Taq) specific sequences of RNA. Using DNA polymerase of *Carboxydotherrnus hydrogenoformans* polymerase a high specificity of transcription is observed with short incubation times. A high specificity is observed using e.g. 5 min of incubation time and 33 units of DNA polymerase protein. With longer incubation times also with lower amounts of *Carboxydotherrnus hydrogenoformans* polymerase specific products can be obtained. However an unspecific smear of products is occurring. These unspecific products might be caused by the 5'-3' exonuclease activity of the polymerase which enables the enzyme to cleave the template at secondary structures ("RNaseH"-activity) and to create additional primers which can be elongated by the DNA polymerase activity. The thermostable DNA polymerase from *Carboxydotherrnus hydrogenoformans* has been identified and cloned and is described in the copending European application with the Application No. 96115873.0, filed October 03, 1996, and incorporated herein by reference.

In summary, reverse transcriptases as MoMULV-RT or AMV-RT perform reverse transcription in the presence of magnesium-ions. However, these enzymes act at temperatures between 37°C and 55°C. Reverse transcription at higher temperatures would be desirable because secondary structures can be overcome in the template in order to avoid premature termination of the reaction and to assure the production of cDNA without deletions.

Other enzymes e.g. DNA polymerase obtainable from *Thermus spec.* act as reverse transcriptase at temperatures up to 70°C in the presence of manganese ions. These reaction conditions are suboptimal, because in the presence of manganese ions the polymerase copies the template RNA with low fidelity and the RNA strand will be degraded. Degradation of the RNA strand occurs faster in the presence of manganese ions as in the presence of magnesium ions. Therefore, if manganese ions are present complexation of the manganese ions (e.g. with EDTA) is

required after cDNA synthesis in order to obtain a higher fidelity during cDNA amplification in the subsequent PCR reaction.

Therefore, it is desirable to develop a reverse transcriptase

- which acts at higher temperatures to overcome secondary structures in the template to avoid premature termination of the reaction and to assure the production of cDNA without deletions
- which is active in the presence of magnesium ions in order to prepare cDNA from RNA templates with higher fidelity and
- which has 3'-5'-exonuclease in order to remove misincorporated nucleotides before continuation of DNA synthesis and to produce products with low mutation frequency
- which has a high specificity and produces exclusively or predominantly RT-PCR products derived from specific primer binding.

The present invention addresses these needs and provides a DNA polymerase mutant active at higher temperatures which has reverse transcriptase activity in the presence of magnesium ions and which has 3'-5' exonuclease activity and reduced or no 5'-3' exonuclease activity.

It is an object of this invention to provide a polymerase enzyme (EC 2.7.7.7.), characterized in that it has reverse transcriptase activity in the presence of magnesium ions as well as in the presence of manganese ions. In a further aspect the invention comprises a DNA polymerase having 3'-5'-exonuclease activity and reduced 5'-3' exonuclease activity. The enzyme according to the invention can be obtained from a polymerase obtainable from *Carboxydo-thermus hydrogenoformans* (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, DSM No. 8979). In a further aspect the invention is directed to a DNA polymerase with reduced 5'-3' exonuclease activity having reverse transcriptase activity in the presence of magnesiums ions and in the substantial absence of manganese ions. In a further aspect the invention comprises a DNA polymerase having a molecular mass of about 64 to 71 kDa as determined by SDS PAGE analysis. The

mutant polymerase enzyme with reduced 5'-3' exonuclease activity derived from a polymerase obtainable from *Carboxidothermus hydrogeniformans* is called hereinafter  $\Delta$  Chy Polymerase. In a further aspect the invention comprises a recombinant DNA sequence that encodes DNA polymerase activity of the  $\Delta$  Chy Polymerase. In a related aspect, the DNA sequence is depicted as SEQ ID No. 10 (Figure 1). In a second related aspect the invention comprises a recombinant DNA sequence that encodes essentially amino acid residues 1 to 607 (SEQ ID No. 11, Figure 1). In a further aspect the invention comprises a recombinant DNA plasmid that comprises the DNA sequence of the invention inserted into plasmid vectors and which can be used to drive the expression of the  $\Delta$  Chy DNA polymerase in a host cell transformed with the plasmid. In a further aspect the invention includes a recombinant strain comprising the vector pDS56 carrying the  $\Delta$  Chy DNA polymerase gene and designated p $\Delta_{2-225}$ AR<sub>4</sub>. The E.coli strain XL1 carrying the plasmid p $\Delta_{2-225}$ AR<sub>4</sub> was deposited on the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig DSM No. 11854 (BMTU 7307) is designated *E.coli* GA1.

In referring to a peptide chain as being comprised of a series of amino acids "substantially or effectively" in accordance with a list offering no alternatives within itself, we include within that reference any versions of the peptide chain bearing substitutions made to one or more amino acids in such a way that the overall structure and the overall function of the protein composed of that peptide chain is substantially the same as - or undetectably different to - that of the unsubstituted version. For example it is generally possible to exchange alanine and valine without greatly changing the properties of the protein, especially if the changed site or sites are at positions not critical to the morphology of the folded protein.

3'-5' exonuclease activity is commonly referred as "proofreading" or "editing" activity of DNA polymerases. It is located in the small domain of the large fragment of Type A polymerases. This activity removes mispaired nucleotides from the 3' end of the primer terminus of DNA in the absence of nucleoside triphosphates (Kornberg A. and Baker T. A. (1992) DNA Replication W. H. Freeman & Company, New York). This nuclease action is suppressed by deoxynucleoside triphosphates if they match to the template and can be incorporated into the polymer.

The 3'- 5' exonuclease activity of the claimed DNA polymerase can be measured as degradation or shortening of a 5'-digoxigenin-labeled oligonucleotide annealed to template DNA in the absence or presence of deoxyribonucleoside triphosphates or on DNA fragments in the absence or presence of deoxyribonucleoside triphosphates.

*Carboxydotherrnus hydrogenofornans* DNA polymerase is the first DNA polymerase isolated from thermophilic eubacteria with a higher activity in the presence of magnesium ions than in the presence of manganese ions as shown in figure 2. The reverse transcriptase activity in dependence of magnesium is of advantage since the DNA polymerases synthesize DNA with higher fidelity in the presence of magnesium than in the presence of manganese (Beckmann R. A. et al. (1985) *Biochemistry* 24, 5810-5817; Ricchetti M. and Buc H. (1993) *EMBO J.* 12, 387-396). Low fidelity DNA synthesis is likely to lead to mutated copies of the original template. In addition,  $Mn^{2+}$  ions have been implicated in an increased rate of RNA degradation, particularly at higher temperatures and this can cause the synthesis of shortened products in the reverse transcription reaction.

The DNA sequence (SEQ ID No.: 10) of  $\Delta$  Chy polymerase and the derived amino acid sequence (SEQ ID No.: 11) of the enzyme are shown in figure 1. The molecular weight deduced from the sequence is 70,3 kDa, in SDS polyacrylamide gel electrophoresis however  $\Delta$  Chy polymerase has an electrophoretic mobility of approx. 65 kDa.

The  $\Delta$  Chy DNA Polymerase has reduced 5'-3' - exonuclease activity and has a temperature optimum at 72°C and exhibits reverse transcriptase activity at temperatures between 50 °C and 75 °C.

When using  $\Delta$  Chy DNA Polymerase obtainable from *Carboxydotherrnus hydrogenofornans* having reduced 5'-3' - exonuclease activity in RT-PCR as reverse transcriptase with subsequent PCR reaction using Taq-polymerase as PCR enzyme a remarkable high sensitivity is achieved (Figure 3). The sensitivity of  $\Delta$  Chy DNA Polymerase in RT-PCR is higher than the sensitivity of e.g. DNA polymerase from *Thermus thermophilus* (Tth polymerase) (Example

3, Figure 4).  $\Delta$  Chy DNA Polymerase also exhibits high sensitivity by amplifying a 1.83 kB fragment from total RNA from human muscle (Figure 5). The error rate of  $\Delta$  Chy DNA Polymerase is  $1.58 \times 10^{-4}$  mutations per nucleotide per cycle and is therewith lower than the error rate of Tth Polymerase which is  $2.37 \times 10^{-4}$  mutations per nucleotide per cycle. This results in higher fidelity of  $\Delta$  Chy DNA polymerase in comparison to Tth Polymerase.

*Carboxydotherrnus hydrogenoformans* was isolated from a hot spring in Kamchatka by V. Svetlichny. A sample of *C. hydrogenoformans* was deposited on the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) under the terms of the Budapest Treaty and received Accession Number DSM 8979. The thermostable polymerase isolated from *Carboxydotherrnus hydrogenoformans* has a molecular weight of 100 to 105 KDa. The thermostable enzyme possesses 5'-3' polymerase activity, a 3'-5'- exonuclease activity and a reverse transcriptase-activity which is  $Mg^{++}$ -dependent. The thermostable enzyme may be native or recombinant and may be used for first- and second-strand cDNA synthesis, in cDNA cloning, DNA sequencing, DNA labeling and DNA amplification.

For recovering the native protein *C. hydrogenoformans* may be grown using any suitable technique, such as the technique described by Svetlichny et al. (1991) *System. Appl. Microbiol.* 14, 205-208. After cell growth one preferred method for isolation and purification of the enzyme is accomplished using the multi-step process as follows:

The cells are thawed, suspended in buffer A (40 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 7 mM 2-mercaptoethanol, 0.4 M NaCl, 10 mM Pefabloc) and lysed by twofold passage through a Gaulin homogenizer. The raw extract is cleared by centrifugation, the supernatant dialyzed against buffer B (40 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 7 mM 2-mercaptoethanol, 10 % Glycerol) and brought onto a column filled with Heparin-Sepharose (Pharmacia). In each case the columns are equilibrated with the starting solvent and after the application of the sample washed with the threefold of its volume with this solvent. Elution of the first column is performed with a linear gradient of 0 to 0.5 M NaCl in Buffer B. The fractions showing polymerase activity are pooled and ammonium sulfate is added to a final concentration of 20 %. This solution is applied to a hydrophobic column containing Butyl-TSK-Toyopearl

(TosoHaas). The column is eluted with a falling gradient of 20 to 0 % ammonium sulfate. The pool containing the activity is dialysed and again transferred to a column of DEAE-Sephrose (Pharmacia) and eluted with a linear gradient of 0-0.5 M NaCl in buffer B. The fourth column contains Tris-Acryl-Blue (Biosepra) and is eluted as in the preceding case. Finally the active fractions are dialyzed against buffer C (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 7.0 mM 2-mercaptoethanol, 100 mM NaCl, 50 % Glycerol).

DNA polymerase activity was measured by incorporation of digoxigenin-labeled dUTP into the synthesized DNA and detection and quantification of the incorporated digoxigenin essentially according to the method described in Höltke, H.-J.; Sagner, G; Kessler, C. and Schmitz, G. (1992) *Biotechniques* 12, 104 -113. The reaction is performed in a reaction volume of 50 µl containing 1 or 2 µl of diluted (0.05 U - 0.01 U) DNA polymerase and 50 mM Tris-HCl, pH 8.5; 12.5 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 10 mM KCl; 5 mM MgCl<sub>2</sub>; 10 mM 2-mercaptoethanol; 33 µM dNTPs; 200 µg/ml BSA; 12 µg of DNase I-activated DNA from calf thymus and 0.036 µM digoxigenin-dUTP.

The samples are incubated for 30 min. at 72°C, the reaction is stopped by addition of 2 µl 0.5 M EDTA, and the tubes placed on ice. After addition of 8 µl 5 M NaCl and 150 µl of Ethanol (precooled to -20°C) the DNA is precipitated by incubation for 15 min. on ice and pelleted by centrifugation for 10 min at 13000 x rpm and 4°C. The pellet is washed with 100 µl of 70% Ethanol (precooled to -20°C) and 0.2 M NaCl, centrifuged again and dried under vacuum.

The pellets are dissolved in 50 µl Tris-EDTA (10 mM/0.1 mM; pH 7.5). 5 µl of the sample are spotted into a well of a nylon membrane bottomed white microwave plate (Pall Filtrationstechnik GmbH, Dreieich, FRG, product no: SM045BWP). The DNA is fixed to the membrane by baking for 10 min. at 70°C. The DNA loaded wells are filled with 100 µl of 0.45 µm-filtrated 1 % blocking solution (100 mM maleic acid, 150 mM NaCl, 1 % (w/v) casein, pH 7.5). All following incubation steps are done at room temperature. After incubation for 2 min. the solution is sucked through the membrane with a suitable vacuum manifold at -0.4 bar. After repeating the washing step, the wells are filled with 100 µl of a 1:10 000-

dilution of Anti-digoxigenin-AP, Fab fragments (Boehringer Mannheim, FRG, no: 1093274) diluted in the above blocking solution. After incubation for 2 min. and sucking this step is repeated once. The wells are washed twice under vacuum with 200  $\mu$ l each time washing-buffer 1 (100 mM maleic-acid, 150 mM NaCl, 0.3 % (v/v) Tween<sup>TM</sup> 20, pH 7.5). After washing another two times under vacuum with 200  $\mu$ l each time washing-buffer 2 (10 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl<sub>2</sub>, pH 9.5) the wells are incubated for 5 min. with 50  $\mu$ l of CSPD<sup>TM</sup> (Boehringer Mannheim, no: 1655884), diluted 1 : 100 in washing-buffer 2, which serves as a chemiluminescent substrate for the alkaline phosphatase. The solution is sucked through the membrane and after 10 min. incubation the RLU/s (Relative Light Unit per second) are detected in a Luminometer e.g. MicroLumat LB 96 P (EG&G Berthold, Wilbad, FRG).

With a serial dilution of *Taq* DNA polymerase a reference curve is prepared from which the linear range serves as a standard for the activity determination of the DNA polymerase to be analyzed.

The Determination of reverse transcriptase activity is performed essentially as described for determination of DNA polymerase activity except that the reaction mixture consists of the following components: 1  $\mu$ g of polydA-(dT)<sub>15</sub>, 33  $\mu$ M of dTTP, 0.36  $\mu$ M of digoxigenin-dUTP, 200 mg/ml BSA, 10 mM Tris-HCl, pH 8.5, 20 mM KCl, 5 mM MgCl<sub>2</sub>, 10 mM DTE and various amounts of DNA polymerase. The incubation temperature used is 50°C.

Isolation of recombinant DNA polymerase from *Carboxydotherrnus hydrogenoformans* may be performed with the same protocol or with other commonly used procedures.

The production of a recombinant form of *Carboxydotherrnus hydrogenoformans* DNA polymerase generally includes the following steps: chromosomal DNA from *Carboxydotherrnus hydrogenoformans* is isolated by treating the cells with detergent e.g. SDS and a proteinase e.g. Proteinase K. The solution is extracted with phenol and chloroform and the DNA purified by precipitation with ethanol. The DNA is dissolved in Tris/EDTA buffer and the gene encoding the DNA polymerase is specifically amplified by the PCR technique using two mixed



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oligonucleotides (primer 1 and 2). These oligonucleotides, described by SEQ ID No.: 1 and SEQ ID No.: 2, were designed on the basis of conserved regions of family A DNA polymerases as published by Braithwaite D. K. and Ito J. (1993) *Nucl. Acids Res.* **21**, 787 - 802. The specifically amplified fragment is ligated into an vector, preferably the pCR<sup>TM</sup>II vector (Invitrogen) and the sequence is determined by cycle-sequencing. Complete isolation of the coding region and the flanking sequences of the DNA polymerase gene can be performed by restriction fragmentation of the *Carboxydotherrnus hydrogenofornans* DNA with another restriction enzyme as in the first round of screening and by inverse PCR (Innis et al., (1990) PCR Protocols; Academic Press, Inc., 219-227). This can be accomplished with synthesized oligonucleotide primers binding at the outer DNA sequences of the gene part but in opposite orientation. These oligonucleotides described by SEQ ID Nos. 3 and 4 , were designed on the basis of the sequences which were determined by sequencing of the first PCR product described above. As template DNA from *Carboxydotherrnus hydrogenofornans* is used which is cleaved by restriction digestion and circularized by contacting with T4 DNA ligase. To isolate the coding region of the entire polymerase gene, another PCR is performed using primers as shown in SEQ ID Nos. 5 and 6. The complete DNA polymerase gene is amplified directly from genomic DNA with primers suitable for introducing ends compatible with the linearized expression vector.

SEQ ID No. 1:

Primer 1: 5'-CCN AAY YTN CAR AAY ATH-3'

SEQ ID No. 2:

Primer 2: 5'-YTC RTC RTG NAC YTG-3'

SEQ ID No. 3:

Primer 3: 5'-GGG CGA AGA CGC TAT ATT CCT GAG C-3'

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SEQ ID NO. 4:

Primer 4: 5'-GAA GCC TTA ATT CAA TCT GGG AAT AAT C-3'

SEQ ID NO. 5:

Primer 5: 5'-CGA ATT CAA TCC ATG GGA AAA GTA GTC CTG GTG GAT-3'

SEQ ID NO. 6:

Primer 6: 5'-CGA ATT CAA GGA TCC TTA CTT CGC TTC ATA CCA GTT-3'

The gene is operably linked to appropriate control sequences for expression in either prokaryotic or eucaryotic host/vector systems. The vector preferably encodes all functions required for transformation and maintenance in a suitable host, and may encode selectable markers and/or control sequences for polymerase expression. Active recombinant thermostable polymerase can be produced by transformed host cultures either continuously or after induction of expression. Active thermostable polymerase can be recovered either from host cells or from the culture media if the protein is secreted through the cell membrane.

The use of a plasmid as an appropriate vector has shown to be advantageously, particularly pDS56 (Stüber, D., Matile, H. and Garotta, G. (1990) Immunological Methods, Letkovcs, I. and Permis, B., eds). The plasmid carrying the Carboxydotherrnus hydrogenofornans DNA polymerase gene is then designated pAR4.

According to the present invention the use of the E. coli strain BL21 (DE3) pUBS520 (Brinkmann et al., (1989) gene 85, 109-114) has shown to be advantageously. The E.coli strain BL 21 (DEB) pUBS 520 transformed with the plasmid pAR4 is then designated AR96 (DSM No 11179).

The mutant  $\Delta$ Chy was obtained by deletion of an N-terminal fragment of the recombinant wild type *Carboxydotherrnus hydrogenoformans* DNA polymerase using inverse PCR (Innis et al., (1990) PCR Protocols; Academic Press, Inc., p 219-227). The reverse primer used is complementary to the cloning site of the expression vector pDS56 (Stüber, D., Matile, H. and Garotta, G. (1990) Immunological Methods, Letkovcs, I. and Pernis, B., eds.) at the Nco I restriction site (bases 120-151) and has the sequence:

SEQ ID No. 7:

Primer 7: 5'-CGG TAA ACC CAT GGT TAA TTT CTC CTC TTT AAT GAA TTC-3'.

This primer contains additional 7 bases at the 5' end to ensure a better binding of the Nco I restriction enzyme in the subsequent restriction enzyme cleavage. The second (forward) primer was complementary to bases 676-702 of the wild type gene and has the sequence:

SEQ ID No. 8:

Primer 8: 5'-CGG GAA TCC ATG GAA AAG CTT GCC GAA CAC GAA AAT TTA-3')

The forward primer also contained an additional Nco I restriction site and additional 7 bases at the 5'-end. Plasmid pDS56 DNA containing the polymerase-gene of *Carboxydotherrnus hydrogenoformans* at the Nco I/BamHI restriction sites was used as template for PCR. The PCR reaction was performed on the circular plasmid DNA pAR4. The fragment encoding the mutated *Carboxydotherrnus hydrogenoformans* DNA polymerase ( $\Delta$  Chy) and the vector DNA were amplified as linear DNA by PCR using the Expand High Fidelity PCR System (Boehringer Mannheim) according to the supplier's specifications (Fig. 7). The length of the gene encoding  $\Delta$  Chy is 1821 bp.

Amplification (Perkin Elmer GenAmp 9600 thermocycler) was carried out with the following conditions:

2 min 94 °C, (10 sec 94 °C; 30 sec 65 °C; 4 min 68 °C) x 10; (10 sec 94 °C; 30 sec 65 °C; 4 min 68 °C) + cycle elongation of 20 sec for each cycle) x 20; 7 min 72 °C;

After PCR the amplified DNA was purified using the High Pure PCR Product Purification Kit (Boehringer Mannheim) and digested with NcoI (3U /  $\mu$ g DNA) for 16 h (Boehringer Mannheim) according to the supplier's specifications.

For extraction with Phenol/Chloroform/Isoamylalcohol (24:24:1) the volume of the sample was raised to 100  $\mu$ l with TE. After extraction the DNA was precipitated by adding 1/10 volumes of 3M Sodium Acetate, pH 5.2 and 2 volumes of EtOH. The DNA was circularized using the Rapid DNA Ligation Kit (Boehringer Mannheim) according to the supplier's specification. The ligated products were introduced into E. coli XL1-Blue by transformation according to the procedure of Chung, C. T. et al. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 2172-2175. Transformants were plated on L-agar containing 100  $\mu$ g/ml ampicillin to allow selection of recombinants. Colonies were picked and grown in L-broth containing 100  $\mu$ g/ml ampicillin. Plasmid DNA was prepared with the High Pure Plasmid Isolation Kit (Boehringer Mannheim) according to the supplier's specification. The plasmids were screened for insertions by digestion with NcoI/BamHI. Strains containing the genes of interest were grown in L-broth supplemented with 100  $\mu$ g/ml ampicillin and tested for the expression of DNA polymerase / reverse transcriptase activity by induction of exponentially growing culture with 1 mM IPTG and assaying the heat-treated extracts (72 °C) for DNA polymerase / reverse transcriptase activity as described above (determination of DNA polymerase activity and determination of reverse transcriptase activity).

The present invention provides improved methods for efficiently transcribing RNA and amplifying RNA or DNA. These improvements are achieved by the discovery and application of previously unknown properties of thermoactive DNA polymerases with reverse transcriptase activity.

The enzyme of this invention may be used for any purpose in which such enzyme activity is necessary or desired. In a particularly preferred embodiment, the enzyme catalyzes reverse transcription of RNA which is amplified as DNA by a second DNA polymerase present in the amplification reaction known as RT-PCR (Powell et al. (1987) *Cell* **50**, 831-840). Any ribonucleic acid sequence, in purified or nonpurified form, can be utilized as the starting nucleic acid(s), provided it contains or is suspected to contain the specific nucleic acid sequence

desired. The nucleic acid to be amplified can be obtained from any source, for example, from plasmids such as pBR322, from cloned RNA, from natural RNA from any source, including bacteria, yeast, viruses, organelles, and higher organisms such as plants and animals, or from preparations of nucleic acids made *in vitro*.

RNA may be extracted from blood, tissue material such as chorionic villi, or amniotic cells by a variety of techniques. See, e.g., Maniatis et al., (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) pp. 280-281. Thus the process may employ, for example, RNA, including messenger RNA, which RNA may be single-stranded or double-stranded. In addition, a DNA-RNA hybrid which contains one strand of each may be utilized.

The amplification of target sequences from RNA may be performed to proof the presence of a particular sequence in the sample of nucleic acid to be analyzed or to clone a specific gene.  $\Delta$  Chy DNA polymerase is very useful for these processes. Due to its 3'-5' exonuclease activity it is able to synthesize products with higher accuracy as the reverse transcriptases of the state of the art.

$\Delta$  Chy DNA polymerase may also be used to simplify and improve methods for detection of RNA target molecules in a sample. In these methods  $\Delta$  Chy DNA polymerase from *Carboxydotherrnus hydrogenofornans* may catalyze: (a) reverse transcription and (b) second strand cDNA synthesis. The use of DNA polymerase from *Carboxydotherrnus hydrogenofornans* may be used to perform RNA reverse transcription and amplification of the resulting complementary DNA with enhanced specificity and with fewer steps than previous RNA cloning and diagnostic methods.

Another aspect of the invention comprises a kit for performing RT-PCR comprising  $\Delta$  Chy polymerase, reaction buffers, nucleotide mixtures, and optionally a thermostable DNA polymerase for detection and amplification of RNA either in a one step reaction or for reverse transcription of the template RNA and subsequent amplification of the cDNA product.

### Brief Description of the Drawings

Figure 1 shows the nucleic acid and amino acid sequence of the "Klenow fragment" of Chy polymerase designated  $\Delta$  Chy.

Fig. 2 shows the reverse transcriptase activity of  $\Delta$  Chy in dependence of magnesium and manganese salt.

Figure 3 shows the reverse transcription and amplification of a 997 bp fragment of the  $\beta$ -Actin gene from total mouse liver RNA using  $\Delta$  Chy and the Expand HiFi-System and decreasing amounts of RNA.

Figure 4 shows the reverse transcription and amplification of a 997 bp fragment of  $\beta$ -actin from total mouse liver RNA in comparison to Tth polymerase. Reverse transcription was either coupled with amplification ("one tube") using the Expand HiFi-System from Boehringer Mannheim, or after reverse transcription the Expand HiFi-System from Boehringer Mannheim was added to the reaction mixture for the subsequent amplification reaction ("two tube").

Figure 5 shows the reverse transcription and amplification of a 1,83 kb fragment of Dystrophin from total human muscle RNA.

Figure 7 shows the reverse transcription and amplification of a 324 bp fragment of  $\beta$ -actin from total mouse liver RNA with various amounts of Chy polymerase and various incubation times.

Figure 7 shows schematically the construction of the clone encoding  $\Delta$  Chy from the clone encoding the wild type gene.

The following examples describe the invention in greater detail:

### Example 1

Reverse transcription of a 324 bp  $\beta$ -actin fragment with Chy wild type DNA Polymerase used as Reverse Transcriptase followed by PCR with Taq-polymerase (Figure 6).

The reaction mixture (20  $\mu$ l) contained 200 ng total mouse liver RNA, 200  $\mu$ M dNTP, 10 mM Tris-HCl, pH 8.8, 5 mM DTT, 10 mM 2-mercaptoethanol, 15 mM KCl, 4.5 mM  $MgCl_2$ , 0.02 mg/ml BSA, 20 pmol of reverse primer ( $\beta$ -actin reverse: 5'-AAT TCG GAT GGC TAC GTA CAT GGC TG-3') and Chy-polymerase 33 units (lanes 1, 4, 7, 10, 13, 16), 13,2 units (lanes 2, 5, 8, 11, 14, 17) and 6,6 units (lanes 3, 6, 9, 12, 15, 18). Reactions were incubated for 5 min (lanes 1 to 6), 10 min (lanes 7 to 12) and 15 min (lanes 13 to 18) at 70 °C.

20  $\mu$ l of the reverse transcription reaction was used as template for PCR (100  $\mu$ l reaction volume) with Taq-polymerase (Boehringer Mannheim) according to the supplier's specification using 20 pmol of forward and reverse primer (Primer sequence " $\beta$ -actin forward": 5'-AGC TTG CTG TAT TCC CCT CCA TCG TG-3', primer sequence " $\beta$ -actin reverse": 5'-AAT TCG GAT GGC TAC GTA CAT GGC TG-3') and 200  $\mu$ M dNTP's.

Amplification was carried out using the following temperature profile:

2 min 94 °C; (10 sec 94 °C; 30 sec 60 °C; 30 sec 72 °C) x 30; 7 min 72 °C

### Example 2

#### Construction of the vector expressing $\Delta$ Chy

The mutant was obtained by deletion of an N-terminal fragment of recombinant wild type *Carboxydotherrmus hydrogenofomans* DNA polymerase using inverse PCR (Innis et al., (1990) PCR Protocols; Academic Press, Inc., p 219-227). The reverse primer used is complementary to the cloning site of the expression vector pDS56 (Stüber, D., Matile, H. and Ga-

rotta, G. (1990) Immunological Methods, Letkovcs, I. and Pernis, B., eds.) at the Nco I restriction site (bases 120-151) and has the sequence: 5'-CGG TAA ACC CAT GGT TAA TTT CTC CTC TTT AAT GAA TTC-3'. This primer contains additional 7 bases at the 5' end to ensure a better binding of the Nco I restriction enzyme in the subsequent restriction enzyme cleavage.. The second (forward) primer, was complementary to bases 676-702 of the wild type gene (sequence: 5'-CGG GAA TCC ATG GAA AAG CTT GCC GAA CAC GAA AAT TTA-3'). The forward primer also contained an additional Nco I restriction site and additional 7 bases at the 5'-end. Plasmid pDS56 DNA containing the polymerase-gene of *Carboxydo-thermus hydrogenoformans* at the Nco I/BamHI restriction sites was used as template for PCR. The PCR reaction was performed on circular plasmid DNA pAR4. The fragment of *Carboxydo-thermus hydrogenoformans* DNA polymerase ( $\Delta$ Chy) and the vector DNA were amplified as linear DNA by PCR using the Expand High Fidelity PCR System (Boehringer Mannheim) according to the supplier's specifications. The length of the gene encoding 1 Chy is 1821 bp.

Amplification (Perkin Elmer GeneAmp 9600 thermocycler) was carried out with the following conditions:

2 min 94 °C, (10 sec 94 °C; 30 sec 65 °C; 4 min 68 °C) x 10; (10 sec 94 °C; 30 sec 65 °C; 4 min 68 °C) + cycle elongation of 20 sec for each cycle) x 20; 7 min 72 °C;

After PCR the amplified DNA was purified using the High Pure PCR Product Purification Kit (Boehringer Mannheim) and digested with NcoI (3U /  $\mu$ g DNA) for 16 h (Boehringer Mannheim) according to the supplier's specifications.

For extraction with Phenol/Chloroform/Isoamylalcohol (24:24:1) the volume of the sample was raised to 100  $\mu$ l with TE. After extraction the DNA was precipitated by adding 1/10 volumes of 3M Sodium Acetate, pH 5.2 and 2 volumes of EtOH. The DNA was circularized using the Rapid DNA Ligation Kit (Boehringer Mannheim) according to the supplier's specification. The ligated products were introduced into E. coli XL1-Blue by transformation according to the procedure of Chung, C. T. et al. (1989) *Proc. Natl. Acad. Sci. USA* 86, 2172-2175. Transformants were plated on L-agar containing 100  $\mu$ g/ml ampicillin to allow selection of recombinants. Colonies were picked and grown in L-broth containing 100  $\mu$ g/ml ampicillin. Plasmid DNA was prepared with the High Pure Plasmid Isolation Kit (Boehringer Mannheim) according to the supplier's specification. The plasmids were screened for inser-



tions by digestion with NcoI/BamHI. Strains containing the genes of interest were grown in L-broth supplemented with 100 µg/ml ampicillin and tested for the expression of DNA polymerase / reverse transcriptase activity by induction of exponentially growing culture with 1 mM IPTG and assaying the heat-treated extracts (72 °C) for DNA polymerase / reverse transcriptase activity as described above (determination of DNA polymerase activity and determination of Reverse Transcriptase activity). (Figure 7)

### Example 3

Reverse transcription and amplification of a 997 bp fragment of β-actin from total mouse liver RNA. Comparison of Δ Chy with Tth polymerase in the reverse transcription reaction (Figure 4) either in a coupled RT-PCR reaction ("one tube") or in consecutive steps, reverse transcription, addition of polymerase and amplification of the cDNA product of the first step.

#### "one tube" system:

The reactions (50 µl) contained 10 mM Tris-HCl, pH 8.8 at 25 °C, 15 mM KCl, 2,5 mM MgCl<sub>2</sub>, 400 µM of each dNTP, decreasing amounts of mouse total RNA (Clontech) as indicated in the figure, 300 nM of each primer, 60 units of Δ Chy and 3,5 units of the Expand HiFi enzyme mix (Boehringer Mannheim GmbH). All reactions were incubated at 60 °C for 30 min (RT step). Amplification followed immediately with following cycle profile (Perkin Elmer GeneAmp 9600 thermocycler):

30 sec. at 94 °C; (30 sec at 94 °C, 30 sec at 60 °C, 1 min. at 68 °C) x 10; (30 sec. at 94°C, 30 sec. at 60°C, 1 min. at 68°C + cyle elongation of 5 sec. for each cycle) x 20; 7 min at 68 °C;

#### "two tube" system:

Reverse transcription is performed in 10 mM Tris-HCl, pH 8.8, 15 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1 % Tween, 4,5 mM MgCl<sub>2</sub>, 2 % DMSO, 800 µM dNTPs, 300 nmoles of each primer, 60 units of Δ Chy, various amounts of total mouse muscle RNA as indicated in the figure. The reaction was performed in a volume of 25 µl for 30 min at 60 °C.

5  $\mu$ l of this reaction are used for the amplification with the Expand HiFi-system from Boehringer Mannheim. Amplification was performed with 2,6 units of polymerase mixture in a reaction volume of 25  $\mu$ l. The following temperature cycling conditions were used: 30 sec. at 94°C, (30 sec. at 94°C, 30 sec at 60°C, 1 min at 68°C) x 10, (30 sec. at 94°C, 30 sec. at 60°C, 1 min at 68°C + cycle elongation for 5 sec for each cycle) X 20.

As a control reaction the same template-primer system was used for RT-PCR with Tth polymerase (Boehringer Mannheim). The reaction was set up according to the supplier's specifications for the "one step" variant.

Claims

- 1) A purified DNA polymerase exhibiting reverse transcriptase activity in the presence of magnesium ions and/or manganese ions having reduced or no 5'-3'-exonuclease activity and substantially no RNaseH activity and obtainable from *Carboxydotherrnus hydrogenoformans*.
- 2) A DNA polymerase as claimed in claim 1 wherein said DNA polymerase exhibits reverse transcriptase activity in the substantial absence of manganese ions.
- 3) A DNA polymerase as claimed in claim 1 or 2 wherein said polymerase exhibits a reverse transcriptase activity which is manganese-dependent.
- 4) A DNA polymerase according to claims 1 - 3 , wherein the magnesium dependent reverse transcriptase activity is higher than the manganese dependent reverse transcriptase activity of said polymerase.
- 5) A DNA polymerase as claimed in any of claims 1-4, wherein said polymerase is a mutant with reduced or no 5'-3' exonuclease activity derived from a naturally occurring polymerase possessing 5'-3' exonuclease activity.
- 6) A DNA polymerase as claimed in any of claims 1-5, wherein said polymerase has an apparent molecular weight between about 64 to 71 kDa as determined by SDS polyacrylamide electrophoresis.
- 7) A recombinant DNA polymerase as claimed in any of claims 1-6, wherein said polymerase is obtainable from *E. coli*, the strain being designated *E.coli* GA1.
- 8) An isolated DNA sequence coding for the polymerase as claimed in any one of claims 1-7.

- 9) A recombinant DNA sequence capable of encoding a DNA polymerase as claimed in any one of claims 1-7.
- 10) An isolated DNA sequence represented by the formula shown in SEQ ID No. 10.
- 11) A vector containing the isolated DNA sequence as claimed in any of claims 8-10.
- 12) A vector according to claim 11, wherein such vector is plasmid pDS56 carrying a deletion mutant of the *Carboxydothemus hydrogenoformans* DNA polymerase gene and is then designated p $\Delta_{2-225}$ AR<sub>4</sub>.
- 13) The vector according to claim 11 providing some or all of the following features:
  - (1) promoters or sites of initiation of transcription
  - (2) operators which could be used to turn gene expression on or off
  - (3) ribosome binding sites for improved translation
  - (4) transcription or translation termination sites
- 14) A microbial host transformed with the vector of claims 11-13.
- 15) A microbial host according to claim 14 wherein said transformant is *E. coli*, the strain being designated *E.coli* GA1.
- 16) A process for the preparation of DNA polymerase according to any of the claims 1-7 comprising the steps:
  - (a) culturing the natural strain *Carboxydothemus hydrogenoformans*
  - (b) suspending the cells of the natural strain in buffer
  - (c) disrupting the cells
  - (d) purifying the DNA polymerase by chromatographic steps including the use of one or more Sepharose-columns.

- 17) A process for the preparation of DNA polymerase according to any one of claims 1-7 comprising growing a recombinant E. coli strain transformed with a vector according to claims 11-13 and purifying and isolating the DNA polymerase.
- 18) A process of amplifying RNA, characterized in that a thermophilic DNA polymerase as claimed in any one of claims 1-7 is used in combination with a thermostable DNA polymerase.
- 19) A process for cDNA cloning and DNA sequencing, characterized in that a thermophilic DNA polymerase as claimed in any one of claims 1-7 is used.
- 20) A process for DNA labeling, characterized in that a thermophilic DNA polymerase as claimed in any one of claims 1-7 is used.
- 21) A process for reverse transcription of RNA to cDNA characterized in that a thermophilic DNA polymerase as claimed in any one of claims 1-7 is used.
- 22) A kit useful for RT-PCR comprising reverse transcription of RNA using a thermophilic DNA polymerase as claimed in any one of claims 1-7 and amplification of the cDNA product by a thermostable DNA polymerase either in a combined reaction (RT and PCR) or for consecutive reactions (RT and subsequently PCR).



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Figure 1:

**Nucleic acid (SEQ ID NO: 10) and protein (SEQ ID NO: 11) sequence of  $\Delta$  Chy DNA polymerase**

```
1   ATGGAAAAGCTTGCCGAACACGAAAATTTAGCAAAAATATCGAAACAATTAGCTACAATC
1   M E K L A E H E N L A K I S K Q L A T I

61  CTGCGGGAAATACCGTTAGAAATCTCCCTGGAAGATTTAAAAGTTAAAGAACCTAATTAT
21  L R E I P L E I S L E D L K V K E P N Y

121 GAAGAAGTTGCTAAATTATTTCTTCACCTTGAGTTTAAAAGCTTTTAAAAGAAATAGAA
41  E E V A K L F L H L E F K S F L K E I E

181 CCAAAAATAAAGAAAGAATACCAGGAAGGTAAAGATTTGGTGCAAGTTGAAACTGTAGAA
61  P K I K K E Y Q E G K D L V Q V E T V E

241 ACGGAAGGACAGATTGCAGTAGTTTTTAGTGATGGATTTTATGTTGATGACGGGGAAAAA
81  T E G Q I A V V F S D G F Y V D D G E K

301 ACAAAGTTTTACTCTTTAGACCGGCTGAATGAAATAGAGGAAATATTTAGGAATAAAAAA
101 T K F Y S L D R L N E I E E I F R N K K

361 ATTATTACCGACGATGCCAAAGGAATTTATCATGTCTGTTTAGAAAAAGGTCTGACTTTT
121 I I T D D A K G I Y H V C L E K G L T F

421 CCCGAAGTTTGTTTTGATGCGCGGATTGCAGCTTATGTTTTAAACCCGGCCGACCAAAT
141 P E V C F D A R I A A Y V L N P A D Q N

481 CCCGGCCTCAAGGGGCTTTATCTAAAGTATGACTTACCGGTGTATGAAGATGTATCTTTA
161 P G L K G L Y L K Y D L P V Y E D V S L

541 AACATTAGAGGGTTGTTTTATTTAAAAAAGAAATGATGAGAAAAATCTTTGAGCAGGAG
181 N I R G L F Y L K K E M M R K I F E Q E
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601 CAAGAAAGGTTATTTTATGAAATAGAACTTCCTTTAACTCCAGTTCTTGCTCAAATGGAG  
201 Q E R L F Y E I E L P L T P V L A Q M E

661 CATACCGGCATTCAGGTTGACCGGGAAGCTTTAAAAGAGATGTCGTTAGAGCTGGGAGAG  
221 H T G I Q V D R E A L K E M S L E L G E

721 CAAATTGAAGAGTTAATCCGGGAAATTTATGTGCTGGCGGGGGAAGAGTTTAACTTAAAC  
241 Q I E E L I R E I Y V L A G E E F N L N

781 TCGCCCAGGCAGCTGGGAGTTATTCTTTTTGAAAACTTGGGCTGCCGGTAATTAAAAAG  
261 S P R Q L G V I L F E K L G L P V I K K

841 ACCAAAACGGGCTACTCTACCGATGCGGAGGTTTTGGAAGAGCTCTTGCCTTTCCACGAA  
281 T K T G Y S T D A E V L E E L L P F H E

901 ATTATCGGCAAAATATTGAATTACCGGCAGCTTATGAAGTTAAAATCCACTTATACTGAC  
301 I I G K I L N Y R Q L M K L K S T Y T D

961 GGCTTAATGCCTTTAATAAATGAGCGTACCGGTAACTTCACACTACTTTTAACCAGACC  
321 G L M P L I N E R T G K L H T T F N Q T

1021 GGTACTTTAACCGGACGCCTGGCGTCTTCGGAGCCCAATCTCCAAAATATTCCCATCCGG  
341 G T L T G R L A S S E P N L Q N I P I R

1081 TTGGAACTCGGTCGGAAATTACGCAAGATGTTTATACCTTCACCGGGGTATGATTATATT  
361 L E L G R K L R K M F I P S P G Y D Y I

1141 GTTTCGGCGGATTATTCCCAGATTGAATTAAGGCTTCTTGCCCATTTTTCCGAAGAGCCC  
381 V S A D Y S Q I E L R L L A H F S E E P

1201 AAGCTTATTGAAGCTTACCAAAAAGGGGAGGATATTCACCGGAAAACGGCCTCCGAGGTG  
401 K L I E A Y Q K G E D I H R K T A S E V



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1261 TTCGGTGTATCTTTGGAAGAAGTTACTCCCGAGATGCGCGCTCATGCCAAGTCGGTGAAC  
421 F G V S L E E V T P E M R A H A K S V N

1321 TTCGGCATTGTTTATGGCATTAGTGATTTTGGTTTAGGCAGAGACTTAAAGATTCCCCGG  
441 F G I V Y G I S D F G L G R D L K I P R

1381 GAGGTTGCCGGTAAGTACATTAAAAATTATTTTGCCAACTATCCCAAAGTGCGGGAGTAT  
461 E V A G K Y I K N Y F A N Y P K V R E Y

1441 CTCGATGAACTTGTCCGTACGGCAAGAGAAAAGGGATATGTGACCACTTTATTTGGGCGA  
481 L D E L V R T A R E K G Y V T T L F G R

1501 AGACGCTATATTCCTGAGCTATCTTCAAAAAACCGCACGGTTCAGGGTTTTGGCGAAAGG  
501 R R Y I P E L S S K N R T V Q G F G E R

1561 ACGGCCATGAATACTCCCCTTCAGGGCTCGGCTGCCGATATTATTAAGCTTGCAATGATT  
521 T A M N T P L Q G S A A D I I K L A M I

1621 AATGTAGAAAAAGAACTTAAAGCCCGTAAGCTTAAGTCCCGGCTCCTTCTTTCGGTGCAC  
541 N V E K E L K A R K L K S R L L L S V H

1681 GATGAGTTAGTTTTAGAAAGTGCCGGCGGAAGAGCTGGAAGAGGTAAAAGCGCTGGTAAAA  
561 D E L V L E V P A E E L E E V K A L V K

1741 GGGGTTATGGAGTCGGTGGTTGAACTGAAAGTGCCTTTAATCGCTGAAGTTGGTGCAGGC  
581 G V M E S V V E L K V P L I A E V G A G

1801 AAAAAGTGGTATGAAGCGAAGTAA  
601 K N W Y E A K \*

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Figure 2:

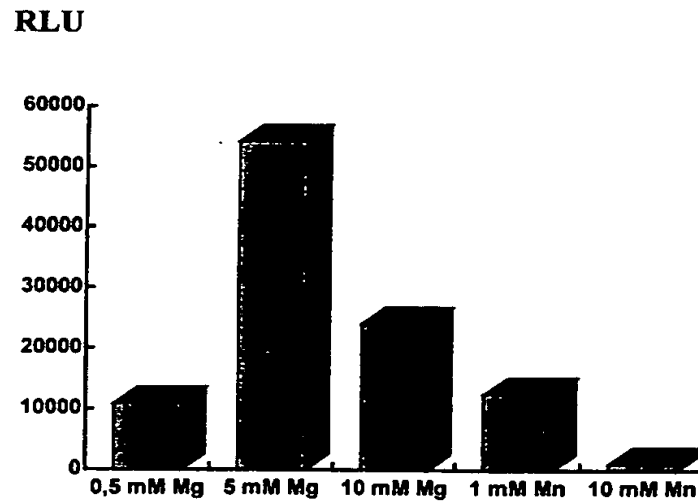
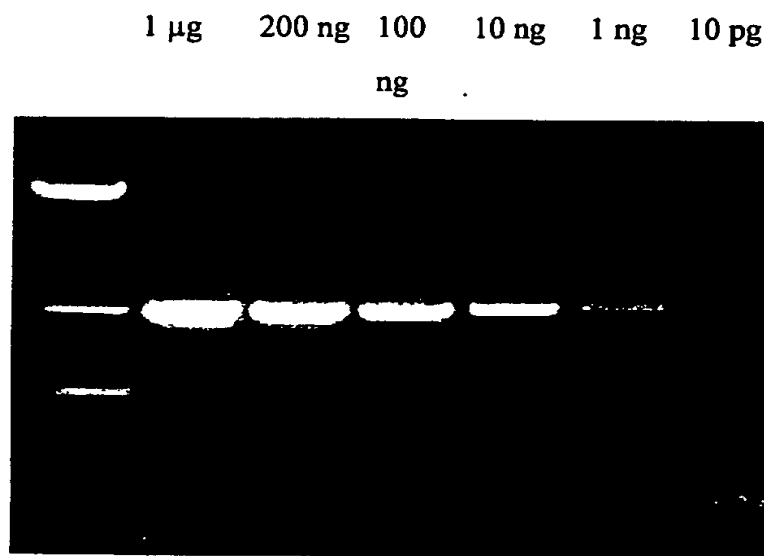


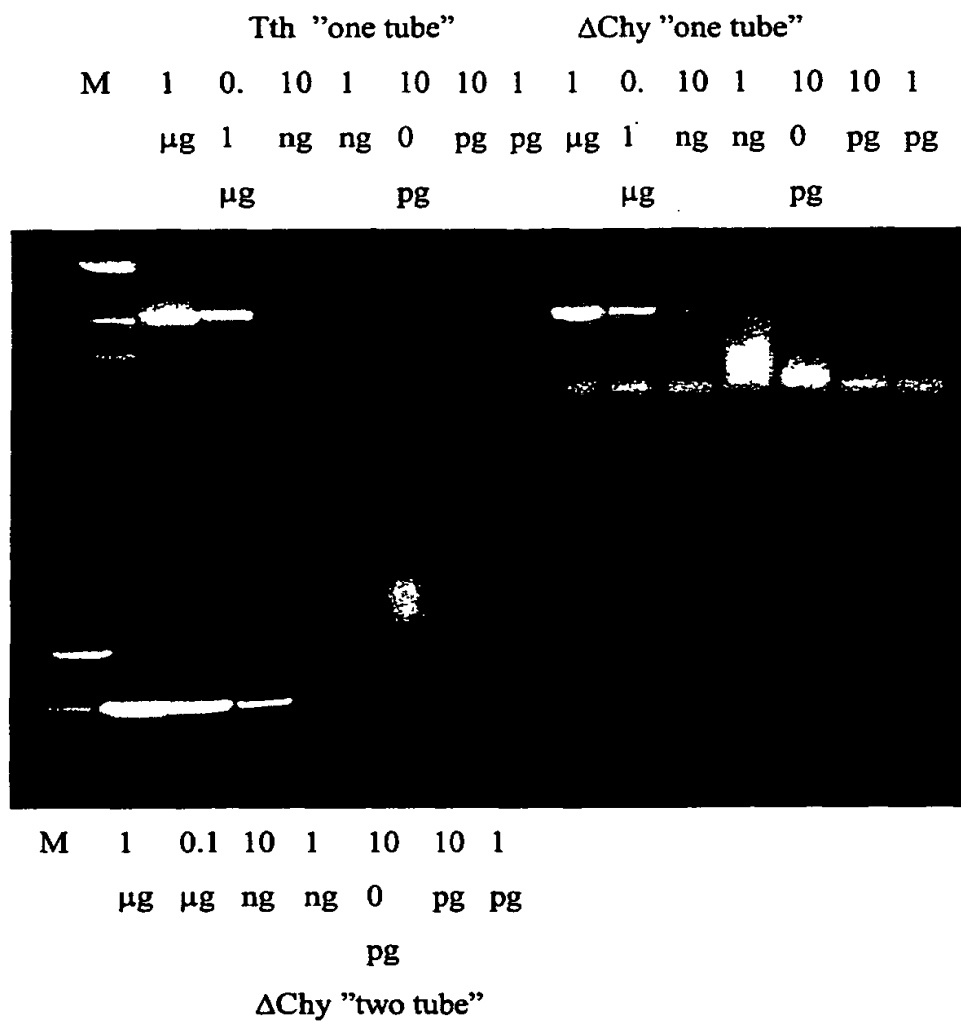
Figure 3:



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Figure 4:



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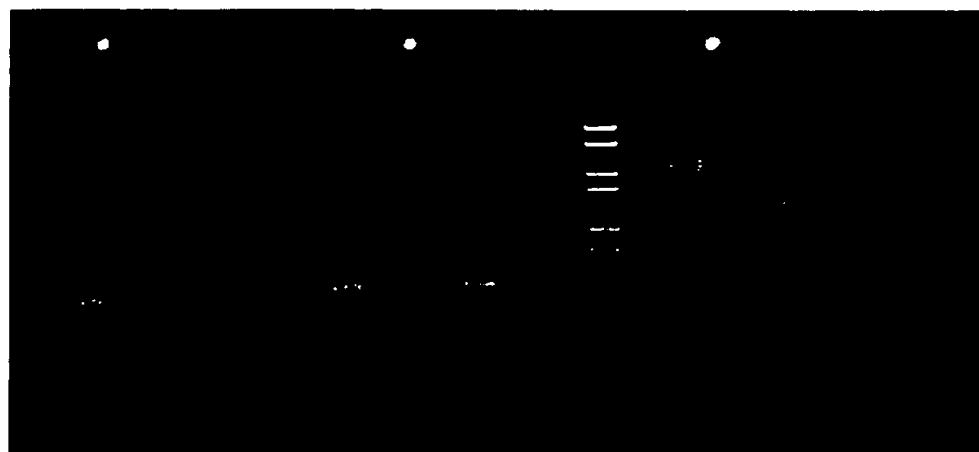
Figure 5:

|   |    |    |    |    |    |    |     |    |   |
|---|----|----|----|----|----|----|-----|----|---|
| M | 50 | 10 | 50 | 10 | 5  | 1  | 500 | 10 | M |
|   | 0  | 0  | ng | ng | ng | ng | pg  | pg |   |
|   |    |    | ng | ng |    |    |     |    |   |



Figure 6:

1 2' 3 4 5 6 7 8 9 10 11 12 M 13 14 15 16 17 18



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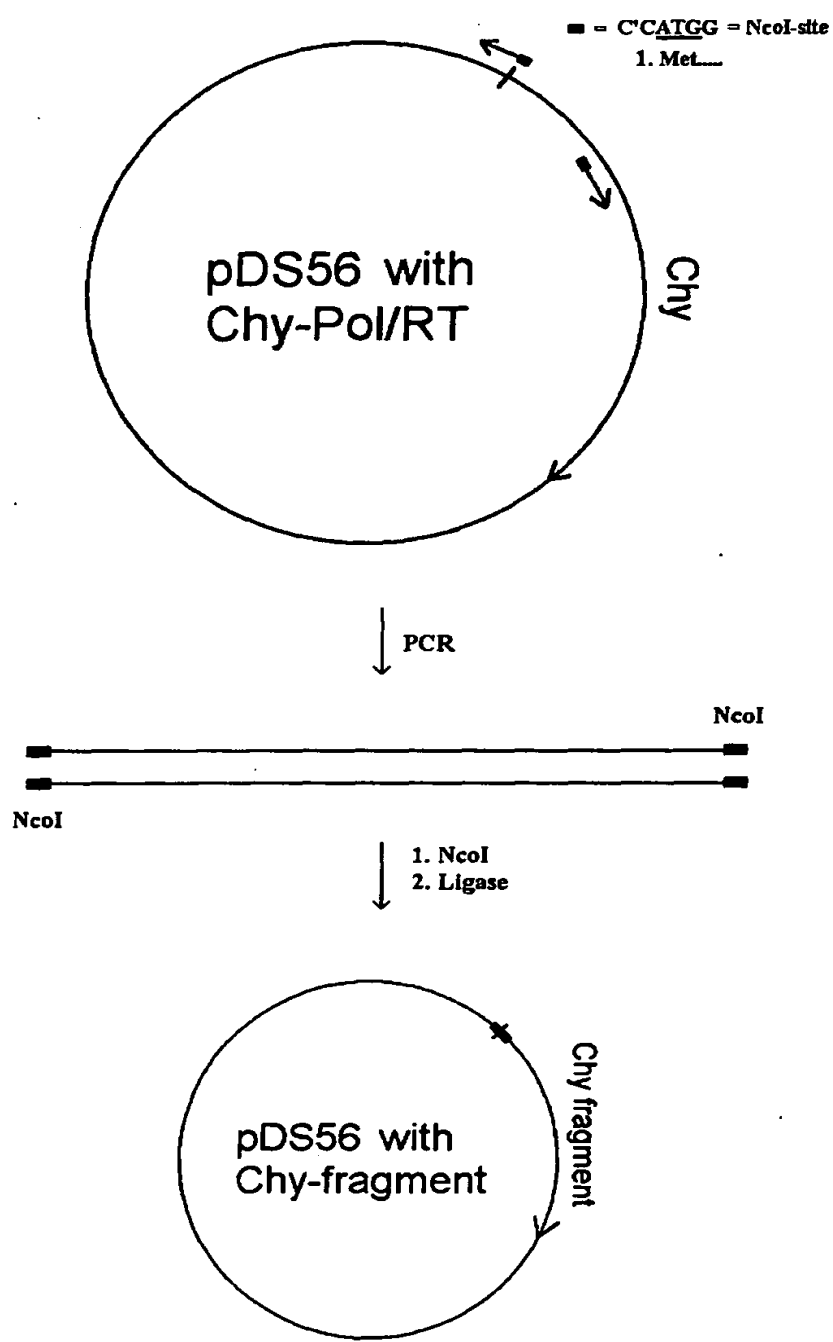


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Figure 7:



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